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(52) UK CL (Edition L)
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G1B BAX
G2F FCE F23E F25A

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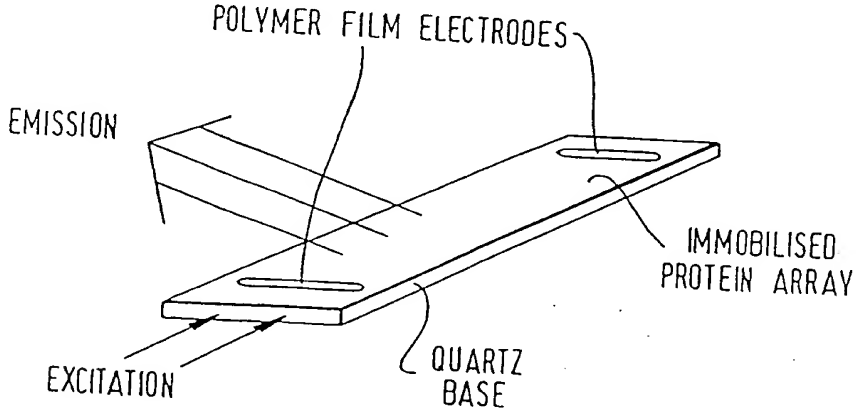
GB 2198738 A EP 0252756 A2 WO 87/01807 A1
US 5089545 A US 5011786 A US 4618916 A

(58) Field of search
UK CL (Edition L) H1K KFX KNA
INT CL[5] H01L
On-line databases: WPI

(54) Biomolecular switch

(57) A biomolecular switch for use in data acquisition/processing devices and in biosensors comprises an array of proteins immobilised on a support such as quartz or aminopropyl glass beads, each protein of the array being capable of reversible interconversion between two or more states. An electrical input is used to modulate the pH and/or ligand concentration (the stimulus) in the microenvironment of the proteins which causes at least some of the protein molecules to selectively convert from a stimulus-free to a stimulus-dependent state. The interconversion between these states is measured by an output means which monitors changes in fluorescence patterns. Alternatively, optical techniques such as a laser pulse can be employed to modulate the microenvironment of the proteins. Other macromolecules such as nucleic acids and polysaccharides may also be used.

FIG. 7



GB 2 266 182 A

FIG. 1

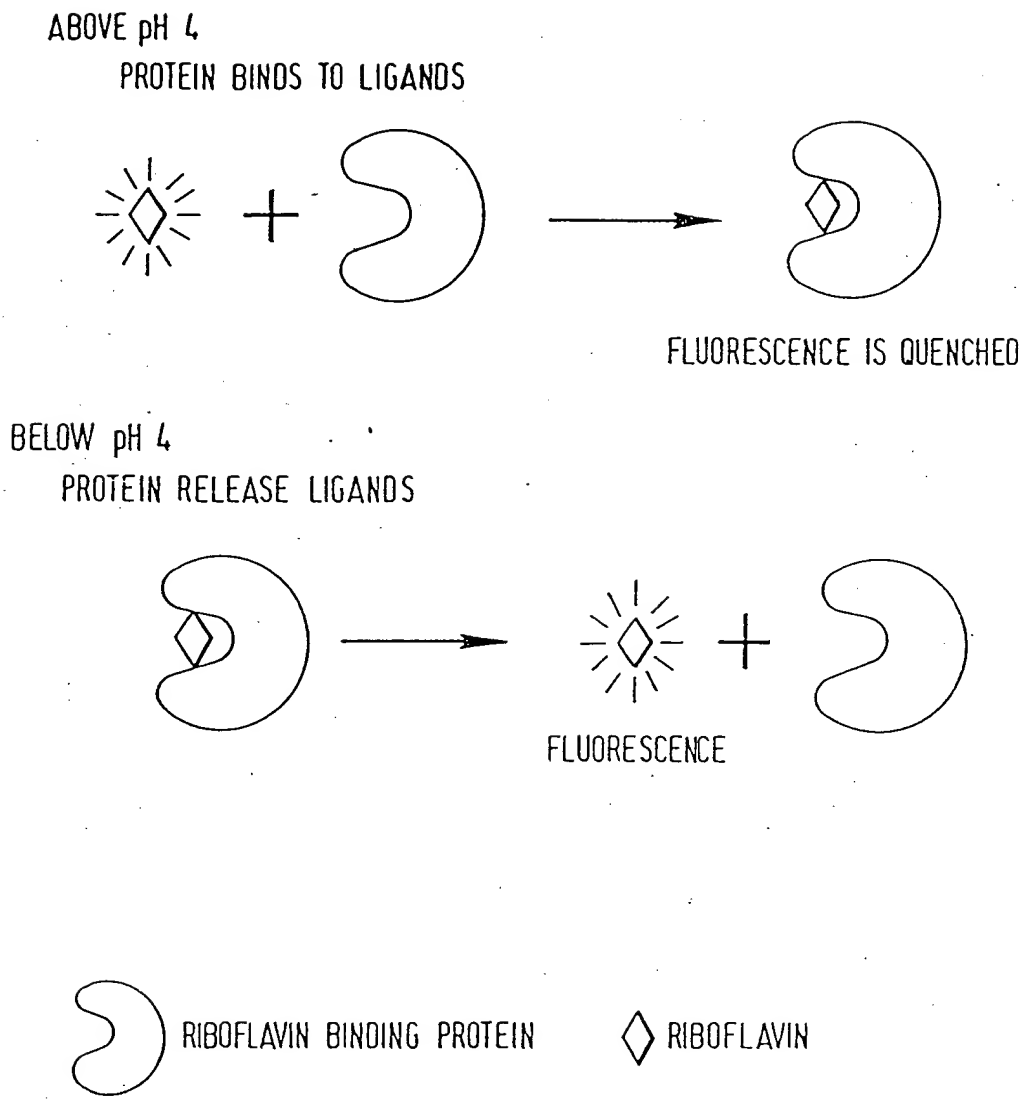

ABOVE pH 4
PROTEIN BINDS TO LIGANDS
FLUORESCENCE IS QUENCHED
BELOW pH 4
PROTEIN RELEASE LIGANDS
FLUORESCENCE
RIBOFLAVIN BINDING PROTEIN
RIBOFLAVIN

FIG. 2

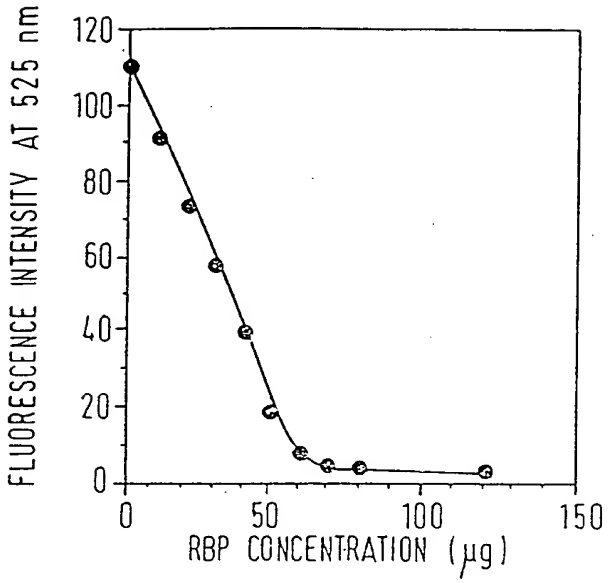

FLUORESCENCE INTENSITY AT 525 nm
120
100
80
60
40
20
0
0
50
100
150
RBP CONCENTRATION (μg)

FIG. 3

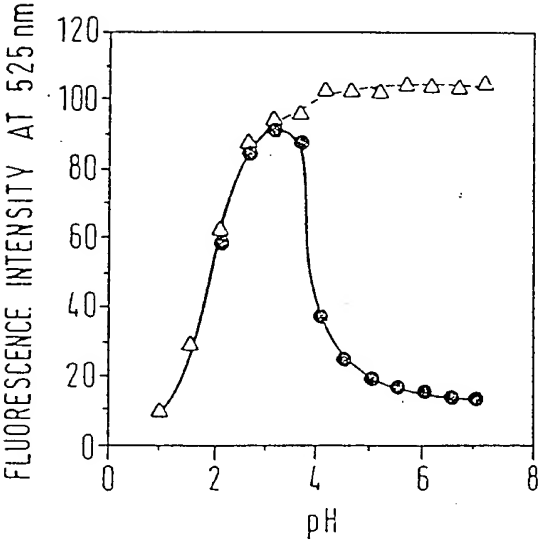
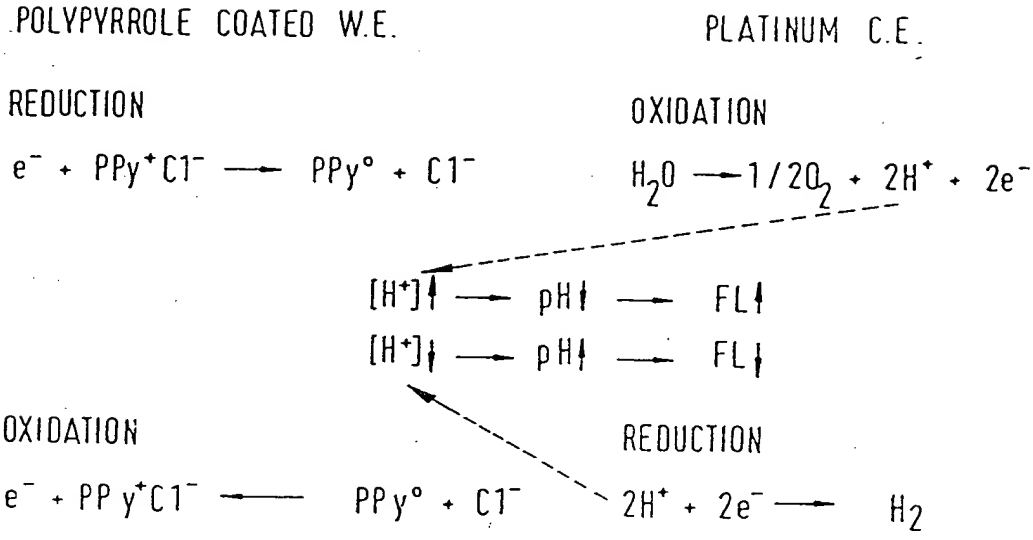

FLUORESCENCE INTENSITY AT 525 nm
120
100
80
60
40
20
0
0
2
4
6
8
pH

FIG. 4



W.E. : WORKING ELECTRODE. C.E. : COUNTER ELECTRODE.
PPy : POLYPYRROLE, FL : FLUORESCENCE

FIG. 5

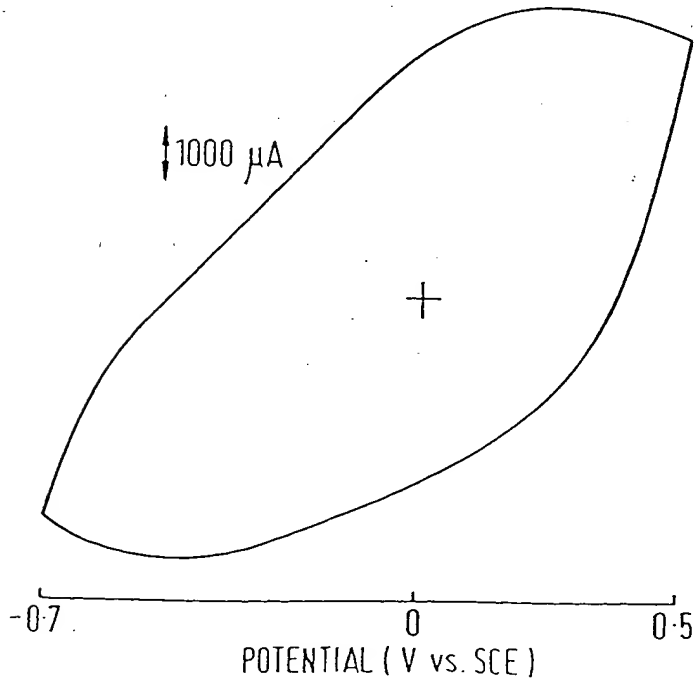


FIG. 6

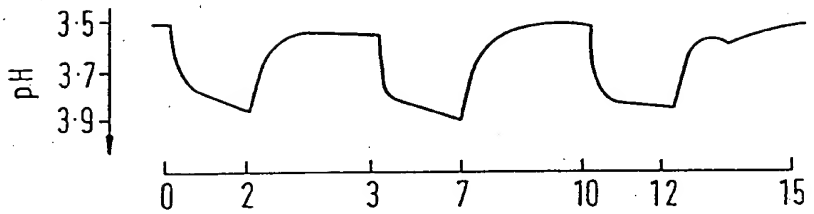

pH
3·5
3·7
3·9
0
2
3
7
10
12
15

FIG. 7

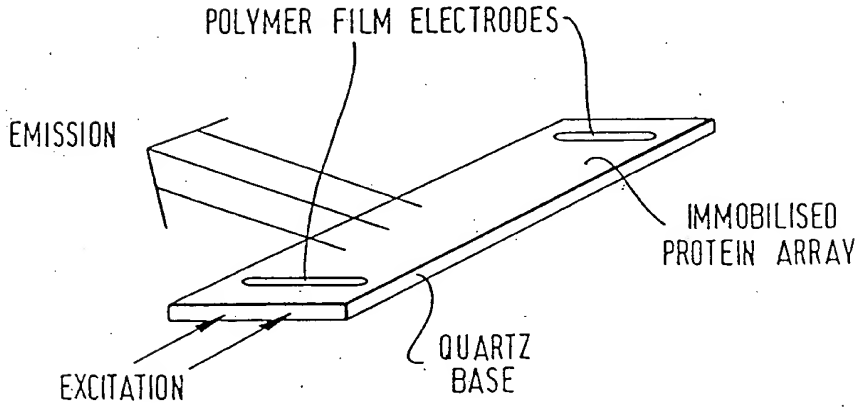

POLYMER FILM ELECTRODES
EMISSION
IMMOBILISED
PROTEIN ARRAY
QUARTZ
BASE
EXCITATION

FIG. 8

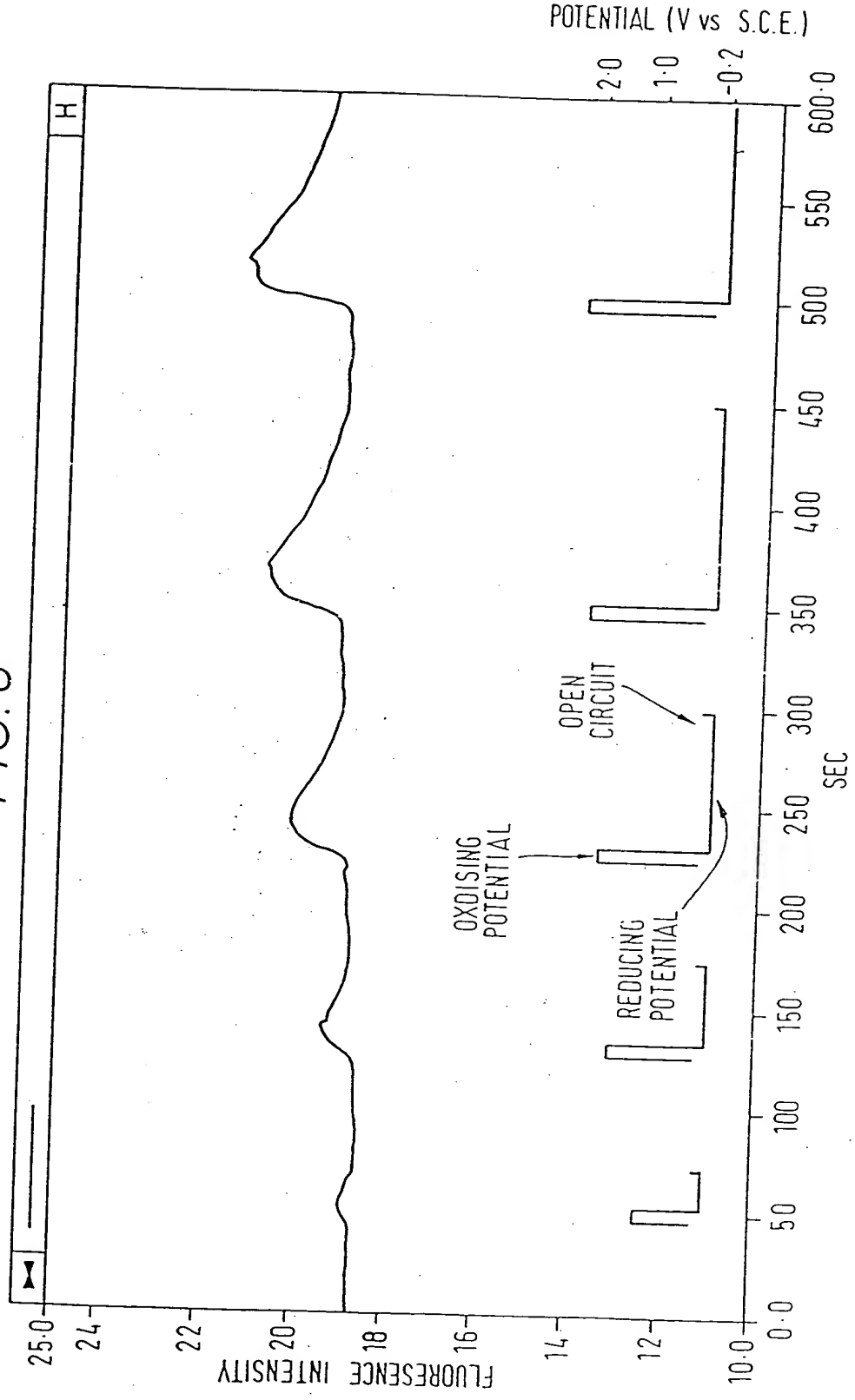

POTENTIAL (V vs S.C.E.)
2·0
1·0
-0·2
H
OPEN CIRCUIT
OXIDISING POTENTIAL
REDUCING POTENTIAL
FLUORESCENCE INTENSITY
25·0
24
22
20
18
16
14
12
10·0
0·0
50
100
150
200
250
300
350
400
450
500
550
600·0
SEC

FIG. 9

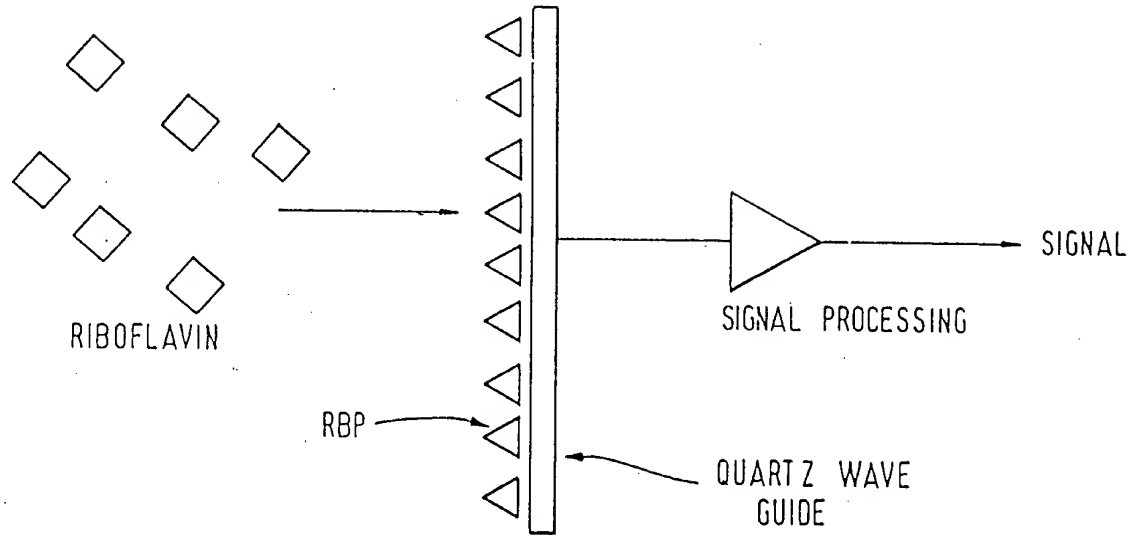

RIBOFLAVIN
RBP
SIGNAL PROCESSING
SIGNAL
QUARTZ WAVE GUIDE

FIG. 10

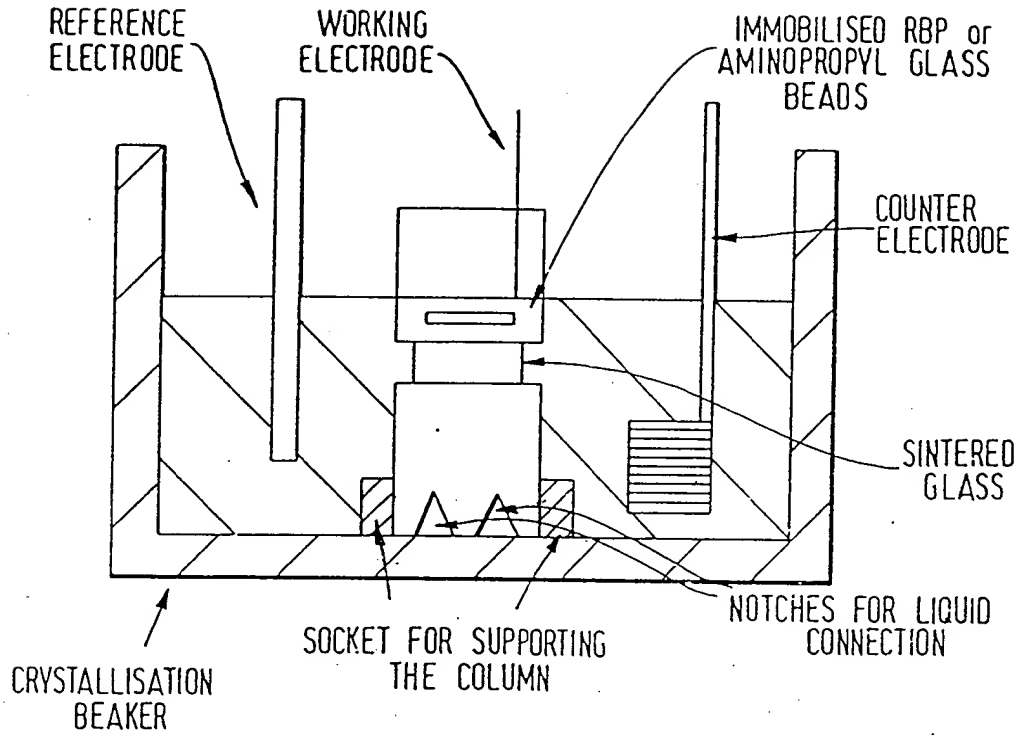

REFERENCE ELECTRODE
WORKING ELECTRODE
IMMOBILISED RBP or AMINOPROPYL GLASS BEADS
COUNTER ELECTRODE
SINTERED GLASS
NOTCHES FOR LIQUID CONNECTION
SOCKET FOR SUPPORTING THE COLUMN
CRYSTALLISATION BEAKER

FIG. 11

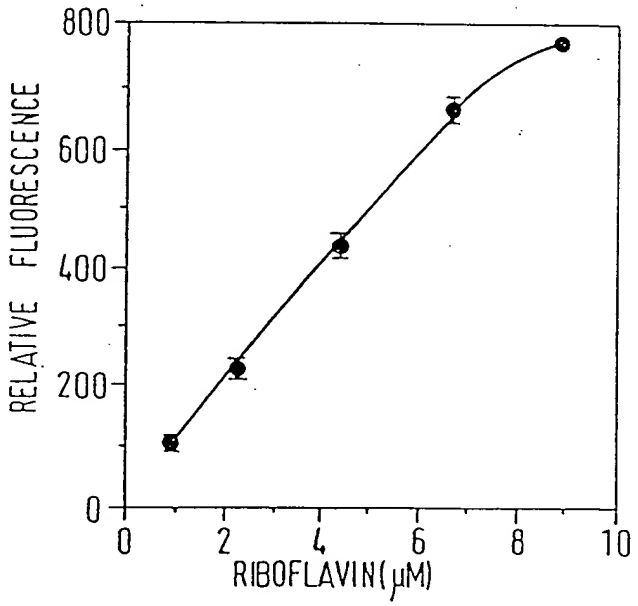

800
600
400
200
0
RELATIVE FLUORESCENCE
0
2
4
6
8
10
RIBOFLAVIN(μM)

FIG. 12

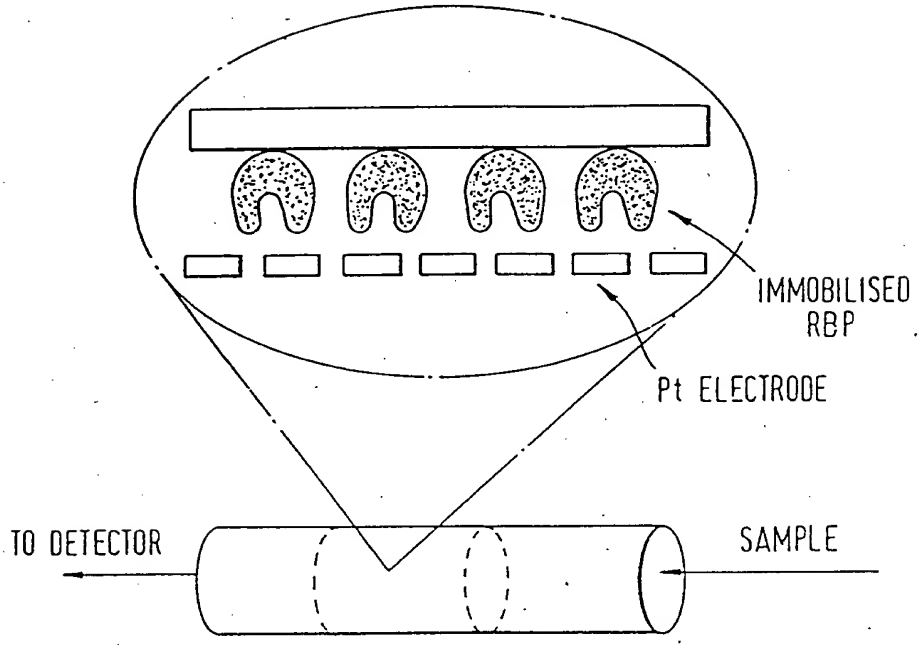
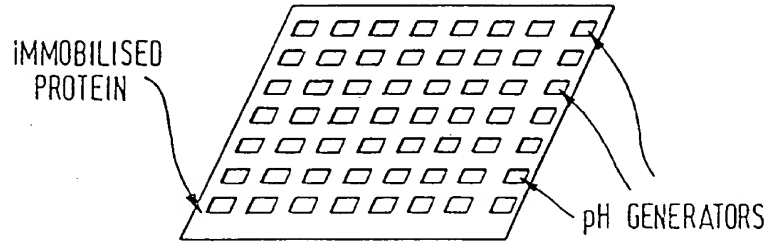

IMMOBILISED
RBP
Pt ELECTRODE
TO DETECTOR
SAMPLE

FIG. 13


IMMOBILISED
PROTEIN
pH GENERATORS

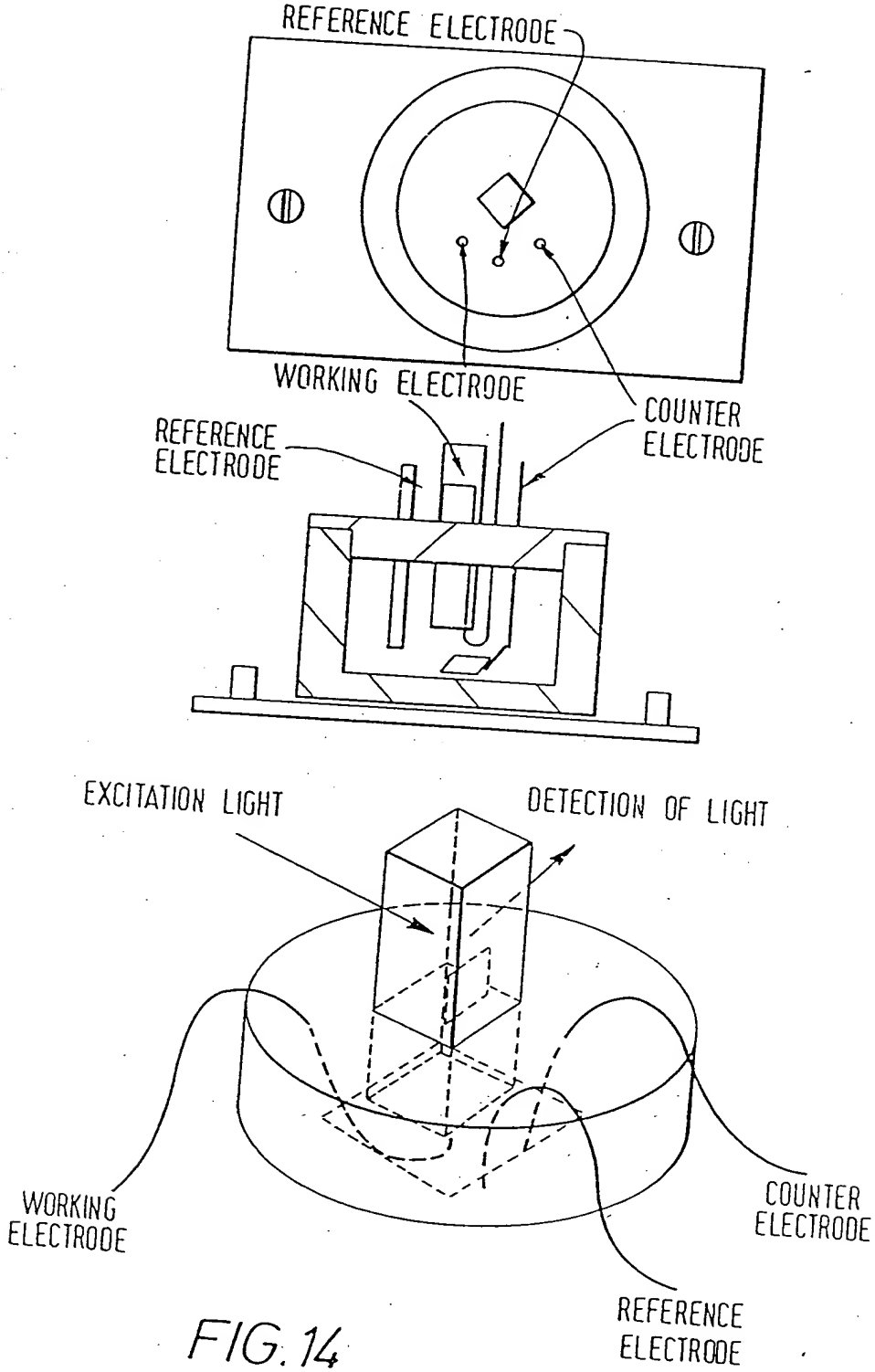

REFERENCE ELECTRODE
WORKING ELECTRODE
REFERENCE ELECTRODE
COUNTER ELECTRODE
EXCITATION LIGHT
DETECTION OF LIGHT
WORKING ELECTRODE
COUNTER ELECTRODE
REFERENCE ELECTRODE

FIG. 14

FIG. 15

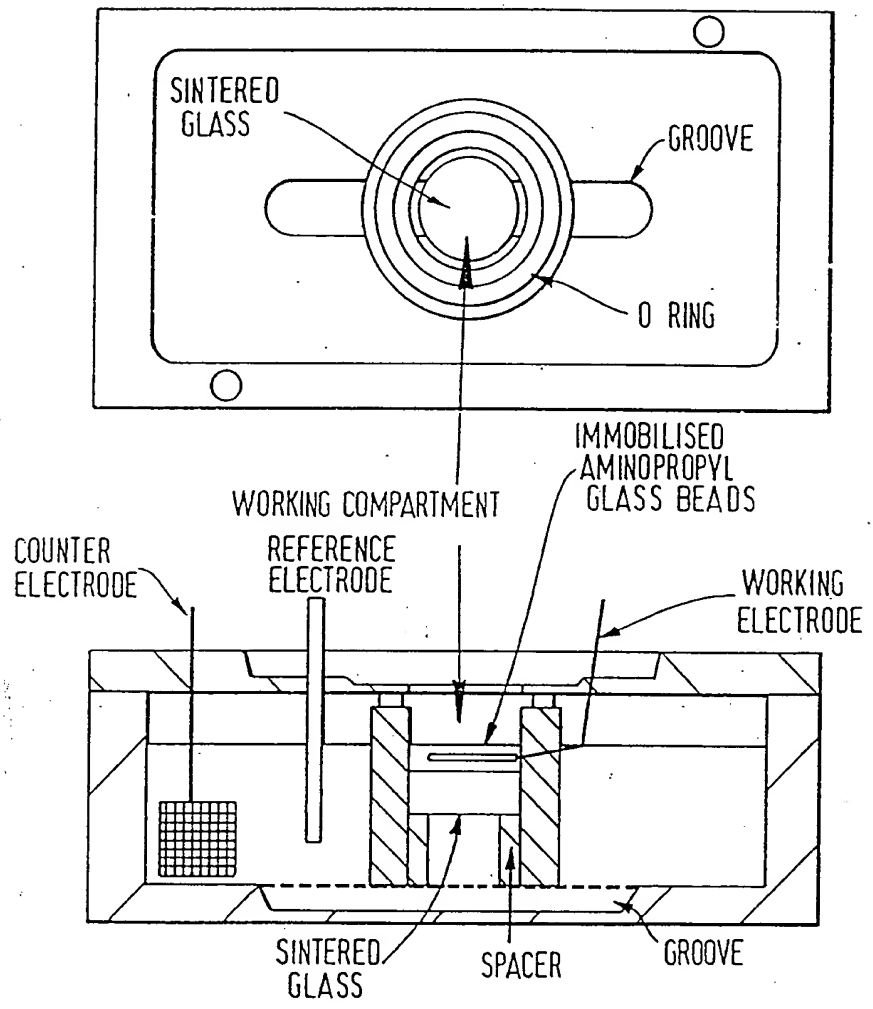
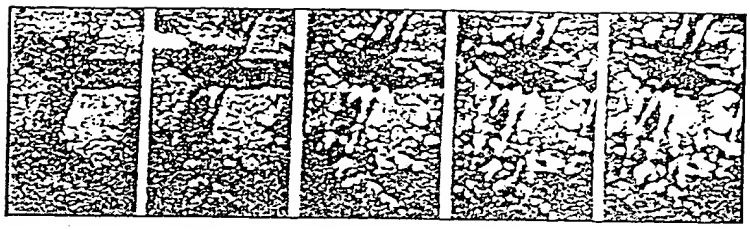

SINTERED GLASS
GROOVE
O RING
IMMOBILISED AMINOPROPYL GLASS BEADS
WORKING COMPARTMENT
COUNTER ELECTRODE
REFERENCE ELECTRODE
WORKING ELECTRODE
SINTERED GLASS
SPACER
GROOVE

FIG. 16

a

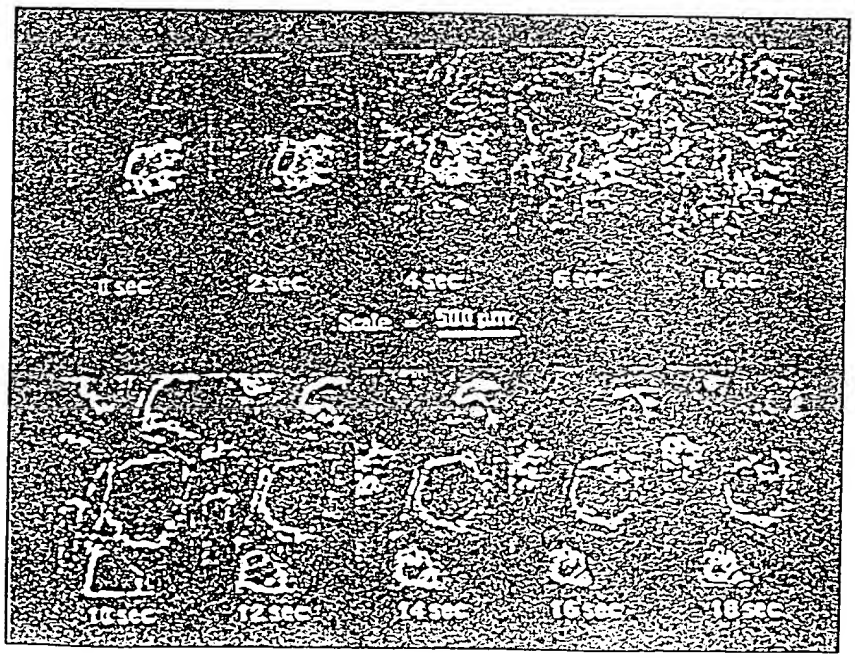


0 Sec 2 Sec 4 Sec 6 Sec 8 Sec

Scale = 500 μm

10 Sec 12 Sec 14 Sec 16 Sec 18 Sec

b



IMPROVEMENTS RELATING TO INFORMATION TECHNOLOGY

This invention relates to the field of information technology and more particularly to bioelectronics and the construction of biomolecular switches using biological macromolecules such as proteins, nucleic acids and polysaccharides for use in data acquisition and processing devices and in biosensors.

Conventionally, digital computers represent variables in a quantised or digital form. Analog variables are, thus, expressed by using one of a number of discrete states or conditions for each value. For example, a decimal system would use the digits 0 to 9. However, ten-state systems, or even multiple-state systems having more than two states when specifically made tend to require a complex arrangement of components and are consequently expensive and are therefore very rare. Therefore, a binary system using only two states represented by 0 and 1 only, is usually employed. Examples of simple two-state systems include mechanical switches (on/off) and transistors (conducting/cut-off). Such systems are usually constructed on very small solid-state circuits consisting of interconnected semiconductor devices printed onto a silicon chip. However, with

the rapid growth in information technology there is an ever increasing demand to improve the level of integration and to produce ever smaller devices for processing information. Although new developments in precision printing technology have enabled smaller devices to be made, the physical limitations (for example, heat dissipation) of the materials commonly used, silicon and gallium arsenide being typical, restrict improvements in integration and reductions in the size of these devices beyond a certain level. The drive for ever smaller structures and ever increasing levels of integration has now led to the discussion of the use of organic materials in the field of molecular electronics and in particular the possibility of using biological macromolecules for such a purpose. For example, Conrad (M) Common ACT 28 464, 1985 describes the use of enzymes in a biomolecular computer which exploits the ability of enzymes to read concentration gradients established by the enzymes' consumption of substrate.

Accordingly, the present invention seeks to provide biomolecular switches which exploit the ability of biological macromolecules to alternate between different states in response to changes in the physical or chemical micro-environment of the macromolecules.

Biological macromolecules have the advantages of being relatively cheap, exhibiting high thermodynamic efficiency and self-assembly properties thereby avoiding some of the synthetic problems posed by the use of organic and inorganic molecules.

Devices based upon such macromolecules have the potential for very high packing density and furthermore provide for the development of non-von-Neumann architectures which has not been hitherto possible with digital computation.

Biological macromolecules such as proteins, nucleic acids and polysaccharides, but more specifically proteins, can exist in two or more states of comparable stability. These states represent differences in their three-dimensional conformation, chemical or physical property, quantum mechanical electronic spin state, and/or biological activity of the macromolecule.

Proteins are of particular utility for the purpose of constructing such biomolecular switches because of their sensitivity to changes in their micro-environment, for example, changes in pH, temperature, ionic strength and ligand concentration can affect the physical and chemical properties of the protein; such changes are commonly manifested by a change in conformation of the protein which is often

coupled with a change in the biological activity of that protein. Such changes in conformation normally occur over a time period of milliseconds.

Clearly, extremes of pH, temperature or ionic strength may result in an irreversible change of state and the degree of perturbation to the protein micro-environment that can be tolerated by the protein is dependent upon the particular protein selected. For example, many proteins are irreversibly denatured at temperatures of 40 to 50°C. However, there are other proteins which exhibit a higher degree of thermal stability and are capable of withstanding elevated temperatures, and such proteins are particularly useful for the construction of biomolecular switches.

The term "micro-environment" in the context of the present invention refers to the local environment of the macromolecule. The term "ligand" used in this context denotes any molecule or ion capable of binding reversibly to a macromolecule. The ligand of choice depends upon the macromolecule selected. However, suitable ligands for use with protein macromolecules include substrates, competitive inhibitors and non-competitive reversible inhibitors; negative and positive effectors; and co-factors.

The affinity of the protein for any particular ligand can be further modulated by external factors

such as pH, ionic strength and temperature of the surrounding micro-environment. For example, a change in pH may effect the ionisation of specific acidic and basic amino acid side chain groups which are vital for the binding of that ligand. Furthermore, the binding event can alter the chemical or physical properties of the ligand also.

Nucleic acids may also be employed as biomolecular switches. Extremes of pH, temperature and ionic strength result in reversible unfolding of double helical DNA, characterised by a decrease in viscosity (this phenomenon being called melting). Return of pH, temperature and ionic strength to normal physiological ranges results in the unwound segments of the two strands being spontaneously annealed or rewound to yield the intact duplex.

The present invention, therefore, seeks to exploit the ability of biological macromolecules to change their physical/chemical properties in response to a specific stimulus or in response to a perturbation of their micro-environment, and to utilise this property to produce biomolecular switches based on these different macromolecular states.

Accordingly, there is provided a biomolecular switch for use in biosensors and data acquisition/processing devices comprising an array of

biological macromolecules immobilised on a support, each biological macromolecule being capable of existing in two or more states of comparable stability, the interconversion between those states being rapid and reversible and being induced by the application or removal of an appropriate external stimulus, thereby permitting the macromolecule to selectively alternate between its stimulus free and stimulus dependant states. In order to exploit this property of macromolecules it is necessary that first, the stimulus can be selectively applied to the micro-environment (the input) and second, the relative populations of the two states can be measured (the output).

The transformation of input to output is modulated by the macromolecule and is dependent on the properties of the particular macromolecule employed. The macromolecule selected can be naturally occurring, synthetic, chemically modified or a product of genetic engineering techniques and includes polysaccharides, proteins such as receptors, enzymes and glycoproteins; and nucleic acids. Preferably the macromolecules used in the fabrication of the biomolecular switches should be pure; however degrees of impurity can be tolerated. Preferred biological macromolecules are those which are available in substantial quantities and which are

thermally stable. Of particular utility in this respect are macromolecules which retain their structure at temperatures in excess of 50°C.

Macromolecules which have been the subject of extensive study and therefore on which detailed structural information is available are particularly useful since that information can readily be used to identify beneficial chemical modifications and to select the appropriate immobilisation technique. Proteins which are useful in the context of bioelectronics are enzymes and receptor proteins involved in cellular message transduction systems.

Any suitable support can be used in the biomolecular switch of the present invention, for example, metal, glass or synthetic polymers can be employed as a support. However, where the input is a change in ligand concentration and therefore the output is dependent upon the number of binding events it is important that the immobilisation of the macromolecule on the support is achieved by techniques which do not utilise functional groups necessary for ligand binding. Various immobilisation techniques are described by L.Q. Zhou and A.E.G Cass Biosensors Bioelec. 6445-450 (1991).

A preferred support is quartz. Quartz offers the advantages that (i) silanisation of the quartz with

silanes carrying organic functional groups offer a variety of routes to immobilise macromolecules, particularly proteins; (ii) metal films can be readily applied thereto prior to the immobilisation of the macromolecules of choice, which films can be used as electrodes to modulate the macromolecular environment; and (iii) when naturally fluorescent or fluorescently labelled macromolecules, particularly proteins, are used and excited, the light emitted from the surface of the quartz support can evanescently couple back into the quartz which allows for efficient collection of light.

A second preferred support is aminopropyl glass beads as their porous structure provides a large surface area and active groups ie amino groups are already present. Glutaraldehyde, carbodiimide and triazine are particularly useful reagents for bridging between the macromolecule and the support.

The nature of the input is, of course, dependent upon the type of macromolecule selected. However, generally it is electrical and that electrical input is used to cause a change in pH, temperature and/or ionic strength of the micro-environment, or to introduce a ligand or modify a constant ligand concentration or any combination thereof. Optionally, the biomolecular switch comprises means for applying

a potential across the array of immobilised macromolecules. The potential applied across the array of macromolecules can be used to change the micro-environment of each macromolecule by modulation of the pH, temperature, ionic strength or ligand concentration thereby effecting a change in the conformation of the macromolecule or the number of binding events which occur. Clearly, the biomolecular switches requiring the use of voltage for operation must contain electrolyte to provide for current flow.

In one embodiment, the biomolecular switch comprises at least one electrode coated with a conducting polymer which can be used to selectively release and take up anions or cations (ligands) from the array; that release/take up being dependent upon the applied potential. Furthermore, the conducting polymer may be used to change $[H^+]$ thereby modulating pH. Clearly, the amount of ligand released or the degree of pH modulation will be dependent upon the period of time for which the voltage is applied and also on the value of the voltage over a certain range of voltages. Reversal of the polarity causes a reversal of the effect.

In such embodiments the effect of an increase in the applied potential is to establish a ligand or pH concentration gradient across the protein array and

such biomolecular switches have particular utility either as a transformation algorithm or a memory wherein an applied voltage (input) is used to control the micro-environment of the protein and the output is a pattern of responses from the macromolecular array modulated by the changes in pattern of stimuli.

In the case where ligand concentration is modulated as described above, the ligand concentration will depend not only on the voltage but also on the existing state of each protein, either liganded or ligand free i.e. the state of ligation of any one protein molecule will depend on the state of ligation of the surrounding molecules. Thus, for any voltage applied for any period of time there will exist a different profile of liganded/unliganded protein molecules.

A change from one state to another can be followed by monitoring variations in the intrinsic properties of the macromolecule and/or ligand, or by tagging the macromolecule or ligand with a detectable label. Any label which is readily detectable can be used and the choice of label will depend largely on the macromolecule selected for fabrication of the biomolecular switch. Methods of labelling macromolecules and ligands are known, for example, M. Brinkley, Bioconjugate Chemistry 3 2-13 (1992) is

illustrative of the range of techniques available.

Methods which can be employed for monitoring changes in state include nuclear magnetic resonance, infrared spectroscopy, UV differential spectroscopy, fluorescence, chemiluminescence and bioluminescence.

According to one aspect of the present invention, the biomolecular switches are employed in a biosensor and act as sensors of the input wherein the output i.e. the relative populations of the two macromolecular states is a direct function of the amount of stimuli applied (input).

According to yet another aspect of the present invention the macromolecule can be used as a sensor of an additional input and simultaneously transform two inputs into a single output wherein the output is a function of the effect of the additional input on the original input-output response.

The invention will now be described by way of example only with reference to the following examples and figures in which:

Figure 1 shows schematically the binding of riboflavin to Riboflavin Binding Protein (RBP);

Figure 2 shows the binding activity of RBP;

Figure 3 shows the effect of pH on the binding reaction;

Figure 4 shows schematically the modulation of pH

using a polypyrrole coated electrode;

Figure 5 shows a typical cyclic voltammogram of a polypyrrole coated electrode;

Figure 6 shows modulation of pH using a polypyrrole coated electrode;

Figure 7 shows a biomolecular switch according to the present invention;

Figure 8 shows the change in riboflavin fluorescence under conditions of potential dependant cycling;

Figure 9 shows a generalised schematic representation of a biosensor according to the present invention;

Figure 10 shows diagrammatically an electrochemical cell suitable for *ex situ* measurements of riboflavin binding and release;

Figure 11 shows the fluorescence intensity of released riboflavin from immobilised RBP by applying a positive potential;

Figure 12 shows a flow cell of immobilised RBP with the platinum electrode;

Figure 13 shows diagrammatically a RBP biomolecular switch in which the pH generating electrodes are arrayed two-dimensionally;

Figure 14 shows the design for a fluorescence spectrochemical cell;

Figure 15 shows the design of an electrochemical cell for the microscope; and

Figure 16 shows fluorescence microscope measurement of riboflavin released from RBP by an applied potential.

EXAMPLE 1: Riboflavin Binding Protein

Riboflavin binding protein (RBP) binds riboflavin (ligand) tightly ($Kd=10^{-9}M$) at pH values above pH 4.5 but releases it readily at a pH value below 4.0. The intrinsic fluorescence of riboflavin is quenched when it is bound to RBP and the fluorescence is only recovered upon release of riboflavin as shown in Figure 1.

The pH of the macromolecular micro-environment is controlled electrochemically by changing the applied potential; in this way the binding of riboflavin to RBP is modulated by an electrical input, and the resultant switching between the two protein states (ligand bound and ligand free) is followed by monitoring a change in fluorescence. In order to control the binding of riboflavin to RBP, it is necessary first to know the binding activity of RBP used and the effect of pH on the binding reaction.

(a) Binding Activity of RBP

A constant riboflavin concentration (0.5μg) was titrated with RBP. Figure 2 shows that approximately

60 μg of RBP was required to quench the fluorescence of 0.5 μg of riboflavin and that the molar ratio (RBP/riboflavin) was 1.3. RBP is known to bind to riboflavin at a ratio of 1:1, the slightly larger molar ratio can be attributed to the impurity of the protein/ligand source or the water absorption of these materials.

Although high purity materials are desirable, lower grade materials can also be used and the above materials were found to be suitable.

(b) Effect of pH on the Binding Reaction

The fluorescence of riboflavin in the presence and absence of RBP (riboflavin and RBP ratio was 1:1.3) was measured at pH values of 2 to 7. The results shown in Figure 3 demonstrate that the change in the fluorescence below pH 3 is independent of RBP but that the change above pH 3 is clearly dependent on the extent of binding of riboflavin to RBP. The pKa of this latter transition was calculated as approximately pH 3.7.

Accordingly, the operating pH of the biomolecular switch comprising RBP is pH 3.7 and is modulated about that pH.

Immobilisation of RBP to silanised glass using, for example, reagents such as triazines, carbodiimides or glutaraldehyde resulted in the protein retaining

its reversible riboflavin binding activity. The source of RBP was egg white and was supplied by STC Laboratories, Winnipeg, Canada. Riboflavin was from yeast and was supplied by Sigma Chemical Co., Poole, Dorset.

(c) pH Modulation of Ligand Binding using a Polypyrrole Electrode

The pH was controlled electrochemically by means of a polypyrrole coated indium tin oxide (ITO) electrode. The use of a polypyrrole coating on the working electrode avoids oxygen and hydrogen evolution which leads to pH changes at both the working and counter electrode. The use of a polypyrrole coated electrode to modulate pH is shown schematically in Figure 4.

The polypyrrole coated indium tin oxide (ITO) electrode is the working electrode and is cut to a size of 8 mm x 50 mm to permit the electrode to fit the inner wall of a fluorimeter cuvette. The counter electrode comprises a platinum mesh 9 mm x 9 mm which provides a large surface area. Polypyrrole is electrodeposited onto the ITO electrode galvanostatically (1 mA/cm^2) from an aqueous solution of 0.1 M pyrrole and 0.1 M HCℓ. Typically electrodeposition is continued for 5 min. The polypyrrole solution is flushed with oxygen-free

nitrogen to remove oxygen from the solution prior to electrodeposition. To confirm that pH could be modulated in this way a polypyrrole coated ITO electrode (working electrode), a platinum mesh electrode (counter electrode) and a Ag/AgCl reference electrode were immersed in 1 M KCl aqueous solution. The solution was also pre-flushed with oxygen-free nitrogen to remove O_2 and CO_2. An ultra thin stem pH electrode was used to monitor pH changes. The potential at the working electrode was then changed between + 0.5V and -0.7 V potentiostatically. Figure 5 which shows a typical cyclic voltammogram of a polypyrrole coated electrode in aqueous solution illustrates that relatively reversible incorporation and release of anions takes place.

The pH change was also controlled when the working electrode and the counter electrode were physically separated. In this case, the potential of the working electrode was maintained above the riboflavin redox potential to avoid the effect of redox reaction, and the solution around the working electrode was separated from the bulk solution where the other working electrodes were located and the incident light beam illuminated only the separate solution. Figure 14 shows diagrammatically the system used when the RBP and riboflavin were dissolved in

solution. The working electrode (Pt mesh) was located in a cuvette which was inserted upside down. The counter electrode (Pt mesh) and reference electrode (Ag/AgCl) were located in a teflon basin in which the bulk was contained. The lid can be rotated to adjust the incident light angle to minimise scattering. When RBP and/or riboflavin were immobilised, the system shown in Figure 15 was used. The working electrode (Pt mesh) and immobilised RBP aminopropyl glass beads were placed in the working compartment at the centre of the cell. The solution in the compartment was separated from the bulk solution with electrical connection through the sintered glass. The depth of the compartment was 0.8mm (which can be altered to 2mm by removing the spacer underneath). A cover glass can be fitted on the centre window of the cell (on the top compartment). The incident light illuminated the working compartment and the change of fluorescence was observed.

The pH was modulated by ± 0.2 units in this way as shown in Figure 6 and such pH changes were used to control RBP-riboflavin binding. The change in riboflavin fluorescence under conditions of potential dependant pH cycling is shown in Figure 8.

EXAMPLE 2: Sulphate and Phosphate Binding Proteins

These proteins are found in the periplasmic space

of Gram-negative bacteria such as E.Coli and S. typhimurium and form part of the nutrient uptake system of those organisms. A variety of molecules such as carbohydrates, amino acids and anions (e.g. phosphate and sulphate) are taken up by these binding proteins and although many examples of these proteins are known they all have a common underlying structure which has been described as "kidney bean". Upon ligand binding the two lobes of the molecule move towards each other burying it. The conformational change is observable both by X-ray diffraction and more importantly in the present example by fluorescence spectroscopy.

(a) Purification and Labelling:

The phosphate binding protein is isolated from E. Coli and the sulphate binding protein from S. typhimurium following published methods (N. Medevsky, H Rosenberg, Biochim. Biophys. Acta 211 158, 1970; and A B Pardee J. Biol Chem 241 5886, 1966 respectively).

A range of fluorescent derivatives of the proteins are prepared using the following fluorophores: fluorescein isothiocyanate which labels amino groups; derivatives of acetamide for cysteine and methionine; and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole which labels tyrosine and lysine. The intrinsic fluorescence of tryptophan residues can also

be measured.

Detailed spectrofluorimetric studies of the different fluorescent derivatives were conducted to determine their response to ligand binding.

(b) Immobilisation:

Platinum metal films are first sputtered onto the quartz to act as electrodes for modulating anion concentration.

Then a particular fluorescent derivative of each of the phosphate binding protein and sulphate binding protein are immobilised on a quartz substrate which has been silanised according to the method of U. Deschler, P. Kleinschmit and P. Pastner, Angew. Chem. (Int. Ed.) 25 236, 1986.

The immobilized fluorescent labelled proteins were then re-characterised by fluorescence microscopy including re-evaluation of ligand binding.

(c) Redox Polymer Films:

The metal films which act as electrodes are coated with polypyrrole by galvanostatic electro-deposition. The polypyrrole conducting polymer exists in two charge states depending upon the applied potential; in the reduced form the polymer is neutral and does not bind ligand (phosphate or sulphate), whereas upon oxidation the polymer is positively charged and binds ligand. The ligand, either

phosphate or sulphate, is present in the protein array at a known concentration. An increase in the voltage applied across the protein array is used to modulate the availability of ligand to each protein molecule by establishing a ligand concentration gradient across the protein array. Release of ligand is achieved at potentials of -0.6 to -0.9 V and uptake of ligand at -0.1 to +0.3 V (all voltages with respect to a saturated calomel electrode). The protein molecules each act as switching elements to produce a fluorescence output from the array of proteins.

This embodiment of biomolecular switch can be used as a transformation algorithm or memory. Figure 7 shows diagrammatically the construction of such a biomolecular switch.

Figure 9 shows a generalised schematic representation of a biosensor in which the immobilised protein array is used as a sensor of the input which can be either an unknown concentration of ligand or a change in pH for example.

A specific example of a biosensor using a riboflavin biomolecular switch is described in detail below.

EXAMPLE 3: - Riboflavin Biosensor

Riboflavin release from an immobilised RBP system can be used as a biosensor for riboflavin because the

binding protein produces an output signal in response to the ligand concentration gradient. For this purpose riboflavin binding protein (RBP) was immobilised on aminopropyl controlled pore glass beads (pore size, 17 nM) obtained from Sigma Chemical Co, using the following method:

(1) the beads were washed three times for two minutes per wash in dichloromethane followed by acetone, ethanol, milli-Q water and dilute detergent and then rinsed thoroughly with milli-Q water;

(2) the beads were washed in 25 % ethanol three times for two minutes and then in absolute ethanol three times for two minutes per wash;

(3) the beads were then incubated at 50 °C for four hours in a solution of triazine in sodium dried toluene (15 ml toluene with 450 μl of 2,4-dichloro-n-propoxy-s-triazine (Lancaster Synthesis) for 300 mg of beads);

(4) the beads were washed five times with toluene and dried under nitrogen;

(5) the beads were then incubated in 4 mg/ml RBP in 0.1M phosphate buffer pH8 with mixing on a rotating mixer at 4 °C overnight ~ 2 days;

(6) the beads were washed at least five times with PBS (0.1 M phosphate buffer with 1.37 M NaCl and 0.027 M KCl) and then three times each with PBS-tween (0.05 % of tween 80) and PBS;

(7) the beads were transferred to 1 M ethanolamine (pH 9) for between two hours and overnight (alternatively 0.1 M Tris/HCl, pH 8 can be used); and

(8) the beads were washed three times with PBS.

Different amounts of riboflavin were mixed with the immobilised RBP aminopropyl glass beads. Then bound riboflavin was released by the procedures described below.

(a) Construction of the Electrochemical Cell

An electrochemical cell was constructed as shown in Figure 10. Immobilised RBP beads were placed in the glass column with a sintered glass filter at the bottom. The working electrode (Pt mesh) was buried in the beads. Notches in the bottom of the column extension allow for liquid connection to the bulk solution and also for the entry of a tube to permit the removal of trapped air. The counter electrode (Pt mesh) and reference electrode (SCE) are located in the bulk solution.

(b) *Ex Situ* Measurement I (Potential controlled riboflavin binding to immobilised RBP)

Riboflavin does not bind to immobilised RBP in acidic solution. However, applying a negative potential (vs SCE) leads to an increase in pH and thus riboflavin binds to immobilised RBP. The Riboflavin-immobilised RBP complex is then transferred to an

acidic solution, releasing riboflavin, whose fluorescence is then measured *ex situ* in the fluorimeter.

At first, the pH change was measured using the following configuration. The working electrode was buried in the immobilised RBP aminopropyl glass beads. The level of the bulk solution (5 μM riboflavin solution in 0.1 M KCl, pH 3) was set to fill the column so that the volume in the column was 0.3ml. The pH measurements were carried out when the potential was at open circuit. By poising the potential at - 0.2 V (vs SCE), the pH change was very small. It took 30 minutes for the pH to increase from 3.0 to 3.5. When the potential was held at -1.0 V, the pH value changed in 5 minutes from 3.0 to 6 or more near the aminopropyl glass beads; and 4.0 after the solution in the column was stirred. Although riboflavin can be reduced at this potential, which can in turn affect the change in fluorescence of riboflavin, the mobility of H^+ is much faster than that of riboflavin so that the pH effect on fluorescence change can dominate over the reduction of riboflavin for a period of up to 5 minutes. To prove this postulate, riboflavin was released in acidic solution (pH 3) after applying the potential at - 1.0 V for 5 to 10 minutes and the fluorescence of released riboflavin was measured. If the redox reaction

dominates the pH change, fluorescence should not be observed. However, fluorescence due to released riboflavin was observed and thus this result indicated that the effect of the redox reaction was not crucial in this measurement configuration.

5 μM riboflavin solution (in 0.1 M KCl, pH 3) was purged with nitrogen for at least 10 minutes. The solution was then poured into the crystallisation beaker and the level was adjusted to fill the glass column to 0.5 ml, where the working electrode and immobilised RBP aminopropyl glass beads were placed beforehand. The potential was held at - 1.0 V for 5 minutes. Then the working electrode was immediately removed and the glass column was connected to a vacuum pump to remove the excess solution in the column. The dried glass beads were then immediately resuspended in 1 ml of pH 3 0.1M KCl solution and the mixture was transferred to a cuvette. After allowing the beads to settle for 2 minutes, the fluorescence of released riboflavin was measured. It was found that removing the solution with a vacuum pump was not enough to remove all unbound riboflavin. Thus, immobilised BSA (bovine serum albumin) on aminopropyl glass beads was used as a control. It had previously been established that the presence of BSA had no effect on the riboflavin fluorescence, even using a 100 fold molar excess with respect to riboflavin. The fluorescence

of riboflavin was then measured with the immobilised BSA using the same procedure as used with the immobilised RBP (see table below).

Immobilised Protein	RBP	BSA
Fluorescence Intensity	188.3±5.8	123.1±1.5

Riboflavin released from immobilised protein on aminopropyl glass beads. After - 1 V was applied in 5 μM riboflavin solution at pH 3, the immobilised proteins were dried and resuspended in 0.1 M KCl solution pH 3. After the aminopropyl glass beads settled, the fluorescence of riboflavin in the supernatant was measured (excitation at 455 nm, emission at 525 nm). Three separate samples for each protein were measured. The difference between RBP and BSA (control) is significant. Thus this result showed that free riboflavin binding to immobilised RBP was controlled by external stimulus, ie the applied potential.

(c) *Ex Situ* Measurement II (Potential controlled riboflavin release from immobilised RBP)

Riboflavin bound to immobilised RBP can be released by a pH decrease resulting from the applying a positive potential (vs SCE). The released riboflavin can then be measured *ex situ*.

Immobilised RBP aminopropyl glass beads were first mixed in 5 μM riboflavin solution at pH 5 to form the riboflavin-RBP complex and were then washed with 0.1 M KCl pH 5 to remove excess riboflavin. The beads were placed in the glass column and the electrode was set as previously described. 0.1 M KCl pH 5 solution was poured into a crystallisation beaker until the column was filled with 1 ml of the solution. It required more than 10 minutes for the pH value to drop below 4 at 2 V (vs SCE). The working electrode was then removed. The column was also removed from the beaker and was left undisturbed for 1 minute to allow the beads to settle. The fluorescence of the supernatant was then measured in the fluorimeter. Fluorescence due to released riboflavin was clearly observed.

Since BSA does not bind to riboflavin, all the riboflavin was removed during the washing stage before applying the potential. Thus the fluorescence was not observed in the supernatant with BSA immobilised on aminopropyl glass beads. This result indicated that riboflavin was released from immobilised RBP by an external stimulus, ie applying positive potential. Since this procedure can be applied to the riboflavin biosensor, the quantitative measurement of this potential controlled riboflavin release is illustrated below.

(d) Biosensor

The applied potential in the riboflavin biosensor was altered to 4 V (vs SCE) in order to increase the measurement speed. At 4 V, 5 minutes was sufficient to effect riboflavin release. Figure 11 shows that the fluorescence intensity of released riboflavin by applying a potential is proportional to the riboflavin concentration in the sample. The fluorescence intensity of released riboflavin excited at 455 nm was then measured at 525 nm.

A linear relationship was observed for sample riboflavin concentrations of 1 - 7 μM. Increasing the total amount of immobilised RBP increases the upper limit of measurement. The lower limit can be improved by an increase in the electrode surface area adjacent to the immobilised RBP beads in order to make the pH change more efficient.

The glass column holding the aminopropyl glass beads and the working electrode can be connected to a tube through which the solution flows, thus the column acts as a flow cell as shown in Figure 12 and permits the continuous measurement of the sample riboflavin concentration.

When the sample solution passes through the column, riboflavin binds to the immobilised RBP column selectively. H^+ is then generated by applying a positive potential to the working electrode to reduce

the pH value and effect the release of riboflavin, which is then measured with a fluorescence detector. The column (sensor) is then ready for the next sample.

RBP can also be immobilised on platinum and ITO electrodes thereby avoiding the need for the aminopropyl glass bead column described above. RBP was immobilised using triazine chemistry (Methods in Enzymology (vol. 44, 1976, Weetall H. H.).

A biosensor developed using this system is reagentless and more importantly it is regenerable *in situ* and therefore reversible. The selectivity can be further improved by using the effect of riboflavin redox on fluorescence if the potential is scanned by another electrode at the detection side. In this way, the presence of interfering materials which may bind to the column non-specifically can be discriminated against.

Riboflavin is vitamin B2 and a biosensor would be useful for measuring its level in urine and blood samples.

Although, the invention has been described by way of examples, it will be apparent that various modifications can be readily made and that the principles applied in the examples are applicable to other biological macromolecules. Furthermore, whilst the above examples focus on biomolecular switches which are homogenous with respect to using a single

macromolecule, clearly, more than one macromolecule/label combination can be used, although it would be necessary to distinguish between the labels, for example, on the basis of the labels having different fluorescence properties.

The electrodes described in the above examples have only been considered in a one-dimensional sense, however, it is clear that these electrodes can be arrayed two-dimensionally, taking advantage of the fact that macromolecules, for example, may sense a two-dimensional concentration profile as shown in Figure 13. This two dimensionally resolved ligand concentration profile controlled by the applied potential was observed by using a fluorescence microscope as shown in Figure 16. The applied potential was + 2V (vs Ag/AgCl) for 10 seconds and then subsequently switched to - 1V. Fluorescence of riboflavin was observed through the filters (excitation 450~490nm, emission 515~585nm) and the image was taken every two seconds by CCD video camera (a) Gray scale images, (b) pseudo colour images.

The pH generating electrodes are arrayed two-dimensionally and the macromolecules are immobilised over the rest of the area with molecular valediction techniques such as a photolithography technique. The array of pH electrodes offers the possibility of complex input patterns. The immobilised

macromolecules are interconnected with each other in the localised areas as well as in the distant area via the concentration gradient pattern and therefore, produce output patterns in fluorescence.

In this arrangement, a group of immobilised macromolecules will be affected not only by immediately adjacent electrodes but also by those at remote sites. Therefore, on a short timescale the macromolecules are strongly affected by the electrodes closest to them, whereas on the longer timescale an effect due to interaction with distant electrodes also occurs. Hence, this array of electrodes offers the possibility of complex input patterns and enhanced performance due to a number of molecules being affected in a parallel fashion dependant on distance from the electrode sites and the timescale of stimulus input.

In this way, the immobilised macromolecules are interconnected with each other in the localised area. Each molecule's binding state affects and is affected by the binding state of its neighbours. Distant macromolecule sites are also interconnected via the effects of the concentration gradient pattern. Due to the multiplicity of input possibilities facilitated by the mutually interacting two-dimensional array structure described above, there is a degree of flexibility of the form in which inputs may be

presented, allowing complex as well as simpler patterns to be considered. On the examples above electrochemical methods have been employed for the generation of response inside the system. However, it is also possible to utilise optical techniques for the modulation of pH, for example, a rapid modulation of pH may be achieved by a laser pulse.

In classical von Neumann theory, questions in the form of input are received and are processed step by step via a series of separate logic operations to arrive at the answer. This kind of procedure is crucially dependant on the type of conventional logic connections and operations that may not be possible in the system envisaged by the biomolecular switch.

A computer using the biomolecular switch of the present invention would act along different lines. The output from this new switch, expressed as two-dimensional binding interaction patterns mediated by pH gradients, is in fact a direct result of the input itself i.e. the posing of the question generates conditions which bring forth the answer as a two-dimensional matrix.

Programming of the system could be facilitated by variations of the binding affinity dynamics which in turn could be accomplished by the use of genetically engineered modified macromolecules or the use of a variety of ligand analogues each with a different affinity for the macromolecule.

CLAIMS:

1. A biomolecular switch suitable for use in a biosensor or a data acquisition/processing device, comprising:

an array of biological macromolecules immobilised on a support, each macromolecule being capable of existing in two or more states between which it can be reversibly interconverted,

input means for enabling a stimulus to be applied to the macromolecules selectively to convert at least some of the macromolecules from a stimulus-free to a stimulus-dependant state, and

output means for measuring or monitoring the changes in state of the macromolecules.

2. A biomolecular switch according to claim 1, wherein the biological macromolecules are proteins, nucleic acids or polysaccharides.

3. A biomolecular switch according to claim 1 or claim 2, wherein the input means is electrical or electromagnetic which serves to modulate the pH, temperature, ionic strength and/or ligand concentration in the microenvironment of the macromolecules.

4. A biomolecular switch according to claim 3, wherein the output means is adapted to measure the relative populations of the stimulus-free and stimulus-dependant states.

5. A biomolecular switch according to claim 3, wherein the input serves to establish a ligand or pH concentration gradient across the macromolecule array, and wherein the output means is adapted to monitor a pattern of macromolecular responses which reflects the change in pattern of applied stimulus.

6. A biomolecular switch according to any one of claims 1 to 5, wherein the means for measuring or monitoring the changes in state of the macromolecules comprises nuclear magnetic resonance, infrared spectroscopy, UV differential spectroscopy, fluorescence, chemiluminescence or bioluminescence.

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Databases (see over)

(i) UK Patent Office

(ii) ONLINE DATABASE: WPI

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Documents considered relevant following a search in respect of claims 1-6

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
X	GB 2198738 A (ALLIED CORP) see page 11	1 at least
X	EP 0252756 A2 (CANON) see page 9 lines 52-55	1 at least
X	WO 87/01807 A1 (HSG VENTURE) whole document	1 at least
X	US 5089545 (BIOTECH INTERNATIONAL) see column 8, lines 39-42	1 at least
X	US 5011786 (MITSUBISHI) see column 2 line 65 column 3 line 18	1 at least
X	US 4618916 (CRONELL RESEARCH) see column 1 line 45 - column 3 line 32	1 at least